

# Wheat Glutenin Subunits. I. Preparative Separation by Gel-Filtration and Ion-Exchange Chromatography<sup>1</sup>

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## ABSTRACT

A combination of gel-filtration and ion-exchange chromatography was used to isolate two pure polypeptide subunits of wheat glutenin for the first time. In native glutenin polypeptide chains are linked by disulfide bonds to form a high-molecular-weight (MW) protein whose viscoelastic properties are important to wheat-flour dough behavior. After reductive cleavage of glutenin disulfide bonds the resulting sulfhydryl groups were alkylated with vinylpyridine to maintain stable polypeptide units. Electrophoretic comparison of reduced glutenin treated with various alkylating agents indicated the derivatives formed by the reaction with vinylpyridine were most suitable for the isolation experiments. Because of the complexity of the subunit mixture, a preliminary separation was made on cross-linked dextran with 0.03M acetic acid-4M urea. It yielded three distinct fractions, A, B, and C, of different MW ranges. Further fractionation of A and C by gel filtration in other systems provided additional resolution. The B and C fractions were chromatographed on sulfoethyl cellulose columns, using an increasing concentration of guanidine hydrochloride for elution. Some eluted peaks contained individual polypeptides while others had only two.

Much of wheat gluten, the protein complex primarily responsible for the rheological properties of flour doughs, can be dissolved in dilute acetic acid. This acetic acid-soluble fraction can be further separated by solvent extraction (1) or by gel-filtration methods (2). Either method gives three fractions: one soluble in saline, consisting of albumins and globulins; gliadin, the 70% ethanol-soluble fraction; and glutenin, the remaining fraction. The hydrated glutenin exhibits strong cohesive-elastic properties in contrast to hydrated gliadin, which appears as a viscous mass. The unique physical properties of glutenin are related to the high molecular weight (MW) of its molecules, which results from covalent linkage of smaller polypeptide chains through disulfide bonds (3). In contrast, gliadin molecules consist of single-chain polypeptides containing only intramolecular disulfide bonds. To aid in further establishing the factors that are responsible for the unique characteristics of native glutenin, its individual constituent polypeptides were isolated to permit determination of their properties.

The disulfide bonds of glutenin are readily cleaved by reducing agents. The resulting sulfhydryl groups in the liberated subunits can be alkylated by a number of vinyl compounds to stabilize the polypeptides against reoxidation to higher weight polymers. The subunits so obtained vary in charge as indicated by starch-gel electrophoresis (4) and in size as indicated by both gel-filtration chromatography (5) and polyacrylamide gel electrophoresis in buffer containing sodium dodecyl sulfate (SDS) (6). Physical and chemical studies on the whole mixture of subunits are complicated by the heterogeneity of the mixture.

Previous efforts to fractionate glutenin polypeptides on a preparative scale have been only partially successful. Crow and Rothfus (5) used gel-filtration

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chromatography to separate cyanoethylated-reduced glutenin (CN-Glu) into three fractions differing in MW. They also partially fractionated the aminoethylated glutenin (AE-Glu) proteins by salt precipitation with cupric ions (7).

The extreme differences in MW, the high degree of aggregation, and the complexity of the mixture appear to be obstacles to satisfactory ion-exchange chromatography of the complete mixture of glutenin polypeptides. In the present investigation, a preliminary fractionation of glutenin subunits by gel-filtration chromatography gave fractions of more uniform MW. Subsequent ion-exchange chromatography of these fractions yielded some pure glutenin subunits suitable for physical and chemical analysis.

## MATERIALS AND METHODS

### Materials

Glutenin protein was extracted with 0.05N acetic acid as previously described (1) from a hard red winter wheat (variety Ponca). Addition of ethanol to 70% by volume and adjustment of the pH to 6.5 precipitated glutenin (1). The precipitated fraction was redispersed in 0.05N acetic acid and reprecipitated to purify it further.

ACS-grade urea from Mallinckrodt Chemical Co. was dissolved in distilled water, passed over a mixed bed resin [AG 501-X8(D) Bio-Rad] to remove cyanates (8) and other trace impurities, and filtered through a 3- $\mu$  Millipore filter. Immediately before use, the solutions were usually degassed under partial vacuum to prevent the formation of air bubbles in gel-filtration columns.

Guanidine hydrochloride (GHCl) came from Eastman Organic (White label). Solutions of GHCl were purified by stirring with activated charcoal (Darco G-60) for 2 hr. and then filtering through a 1.2- $\mu$  Millipore filter. The concentration of GHCl in solution was adjusted to a desired value based on its refractive index (9).

### Sample Preparation

Glutenin disulfide bonds were reduced by treating a 1 to 2% protein solution in 8M urea-*tris*-buffer, pH 7.5, with 2-mercaptoethanol (Eastman Organic) (about 100 molar excess over total disulfides) with stirring for 2 hr. at room temperature. The reduced protein in solution was reacted for 25 min. with a 1:1 molar ratio of acrylonitrile (Aldrich Chem. No. 11,021-3) to the initial 2-mercaptoethanol, like the method of *S*-cyanoethylation by Cavins and Friedman (10). The pH of the solution was adjusted to 3 with acetic acid, and the solution was dialyzed and lyophilized to obtain the modified protein, CN-Glu. The AE-Glu was prepared by the procedure of Rothfus and Crow (7). The *S*-(4-pyridylethyl)glutenin (PE-Glu) was prepared by reacting the reduced protein in the reduction medium with a 1:1 molar ratio of 4-vinylpyridine (Aldrich Chem. No. V320-4) to all sulfhydryl groups, including those in residual mercaptoethanol, for 2 hr. according to the method of Friedman et al. (11).

### Electrophoresis

Starch-gel electrophoresis was conducted as previously described (12), except for the use of 4M urea and pH 3.25 buffer. The electrophoretic properties of the reduced and alkylated glutenins were more sensitive to small compositional changes in the urea-aluminum lactate buffer than were the gliadins (12). After standing for

only 1 day, the buffer used to prepare the starch gels would deteriorate to the extent that protein migration during electrophoresis was decreased and the bands were not as well separated. Therefore, the buffer was prepared immediately before making the starch gels.

SDS-polyacrylamide gel electrophoresis was carried out as described by Bietz and Wall (6).

#### Gel Filtration

Cross-linked dextran gels, Sephadex G-100 and G-200 (Pharmacia), were prepared and poured as recommended by the manufacturer. The column supports were either  $2.5 \times 100$  cm. or  $5.0 \times 100$  cm. glass tubes fitted with adjustable flow adapters. Columns were operated with an upward flow. From 50 to 75 mg. sample was placed on a  $2.5 \times 70$ -cm. column and for the larger preparative runs, up to 250-mg. sample was fractionated on a  $5.0 \times 90$ -cm. column.

Samples were dissolved to give solutions containing approximately 1 to 2% protein by weight. The solutions were centrifuged in a desk-top clinical centrifuge before chromatography to remove any insoluble materials in the sample. The columns were run at 2.0 to 2.5 ml. per  $\text{cm}^2$  per hr. at room temperature. The flow was controlled either by height of the buffer above the outlet with a Minot-type container or by a peristaltic pump. The latter gave a less variable flow rate. The column effluent absorbance was monitored automatically at 280 nm. by a Vanguard recording flow spectrophotometer, or else the absorbance at 280 nm. of the contents of each tube was measured with a Beckman DU spectrophotometer. The desired fractions were combined, dialyzed against 0.1N acetic acid, and lyophilized. The dried samples were weighed to determine approximate recoveries.

#### Ion-Exchange Chromatography

Sulfoethyl cellulose (SEC) (Bio-Rad Laboratories) was used for ion-exchange chromatography as previously described (12). Some fines had to be washed out of the material to gain a proper flow rate. However, removal of all fines tended to give

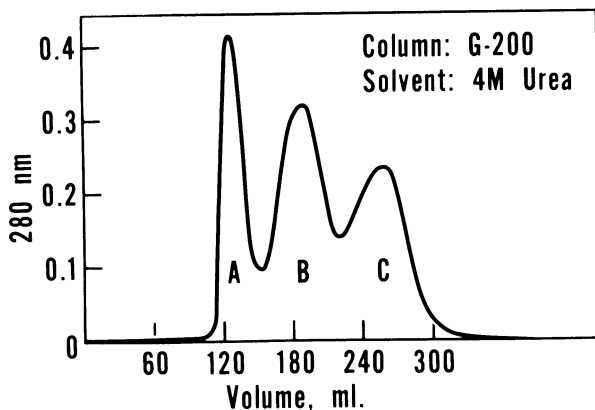


Fig. 1. Gel filtration of pyridylethyl glutenin (PE-Glu) from Ponca wheat. Column: Sephadex G-200,  $2.5 \times 72$  cm.; solvent: 4M urea, 0.03N acetic acid; 50-mg. sample.

TABLE I. PROTEIN RECOVERY FROM CHROMATOGRAPHIC FRACTIONATION OF PONCA PE-GLU<sup>a</sup>

Fraction	Initial Gel-Filtration Chromatography		Rechromatography by Gel Filtration and SEC		
	% PE-Glu recovered <sup>b</sup>	Data based on figure	Subfractions	% Fraction recovered <sup>c</sup>	Data based on figure
A	23	1	A-1	25	8
			A-2	35	
			A-3	10	
			Total	<u>70</u>	
B	31	1	B-0	8	4
			B-1	13	
			B-2	30	
			B-3	9	
			B-4	8	
			B-5	12	
			Total	<u>89</u>	
C	36	1	HC-0	15	7
			HC-1	3	
			HC-2	4	
			HC-3	3	
			HC-4	11	
			HC-5	14	
			Total	<u>60</u>	

<sup>a</sup>PE-Glu = Pyridylethyl glutenin; SEC = sulfoethyl cellulose;  
HC = High-molecular weight C fraction (44,000).

<sup>b</sup>Average by weight recovered  $\pm 3\%$ .

<sup>c</sup>Average by weight and absorbance measurements  $\pm 3\%$ .

too rapid a flow rate with a low back pressure, and resolution of the eluting proteins was impaired. Column dimensions were 1.9 X 50 cm. with a flow rate of approximately 50 ml. per hr. at approximately 5 lb. pressure.<sup>2</sup> The gradient of increasing GHCl concentration was produced by using four chambers of a Buchler Varigrad device. The flow rate was maintained with a Beckman Accu-Flo pump.

## RESULTS

### Selection of Alkylating Agent

Previous workers have alkylated reduced glutenin either with acrylonitrile to yield CN-Glu (5), or ethyleneimine to form AE-Glu (7). Both products present some problems in manipulation and characterization. Cavins and Friedman (10) found that reaction conditions must be carefully controlled to prevent modification of the  $\epsilon$ -amino lysine in proteins by acrylonitrile. Also, because the charges were similar, many of these reduced proteins were not well resolved upon gel electrophoresis. Rothfus and Crow (7) found that AE-Glu was more soluble than

<sup>2</sup>If chromatographic equipment to withstand moderate pressures, a pressure gauge, and an automatic pressure-sensitive switch are available, the column can be operated at 10 to 20 lb. pressure to achieve better resolution.

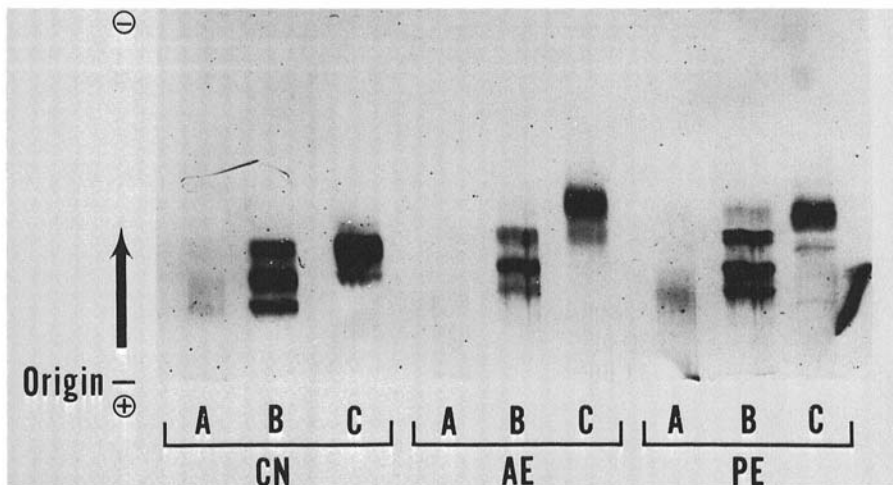


Fig. 2. Starch-gel electrophoresis of three fractions of derivatized reduced glutenins obtained by gel-filtration chromatography. a, Cyanoethyl glutenin (CN-Glu); b, aminoethyl glutenin (AE-Glu); and c, PE-Glu.

CN-Glu and yielded gel patterns with greater differences in the mobilities of the protein derivatives.

The recent introduction of 4-vinylpyridine as an improved alkylating agent for reduced proteins (11) prompted trial studies of it with glutenin. Data from gel-filtration separation of reduced and alkylated glutenin prepared with vinylpyridine are plotted in Fig. 1. The chromatography on Sephadex columns of PE, AE, and CN derivatives of reduced glutenin yielded essentially the same elution profiles. Recovery from the column is given in Table I. Each preparation was resolved into three peaks with different MW ranges designated A, B, and C in similar proportions. Thus each alkylating agent served to stabilize the cleaved protein fragments adequately. As shown in Fig. 2, starch-gel electrophoretic patterns indicate that both the AE- and PE-Glu samples have greater mobilities and exhibit better separation than the CN-Glu sample owing to the additional charge introduced by the alkylating agents. The PE-Glu has the most clearly defined bands, permitting easier identification of the principal components after separation. Furthermore, the vinylpyridine does not react with lysine and gives better half-cystine recovery during amino acid analysis (11). For these reasons vinylpyridine was used as the alkylating agent in subsequent experiments.

#### Molecular Weights of Subunits

The molecular weights of the different components of reduced alkylated glutenin may be evaluated from their elution volumes upon Sephadex chromatography and by SDS electrophoretic analysis of the fractions. The constituents contained in fraction A from the Sephadex G-200 column (Fig. 1) must be of high MW or highly aggregated in the solvent because they are eluted at the void volume. Sephadex G-200 has an exclusion limit between 200,000 and 800,000 MW depending on the configuration of the protein.

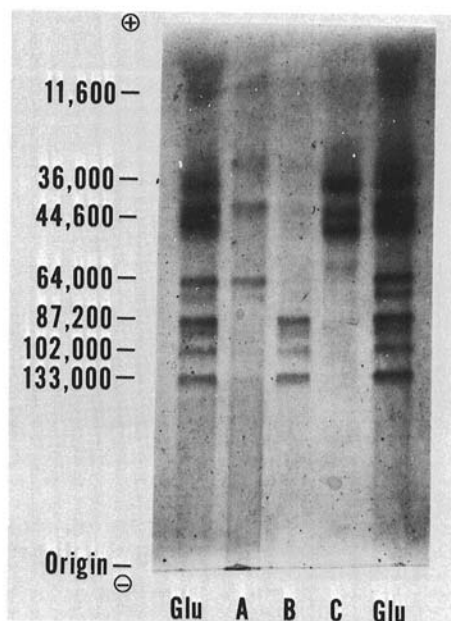


Fig. 3. Sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis of PE-Glu fractions A, B, and C from Fig. 1. Molecular weight (MW) of reference proteins shown at left side (from Ref. 6); (Glu) whole PE-Glu sample.

As shown in Fig. 3, the SDS electrophoresis pattern of the A fraction of PE-Glu exhibits not only considerable streaking, but also bands whose mobilities, by comparison with known proteins, indicate MWs around 60,000, 44,000, 30,000, and lower. The B fraction contains the highest-MW subunits ranging from 87,000 to 133,000 (Fig. 3). The C fraction consists of three main groups having MWs around 37,000, 43,000, and 45,000. Some components of the A fraction are similar to, and some are even lower in apparent MW in SDS media than, subunits of the C fraction as seen in the electrophoretic patterns in Fig. 3. Based on the MWs determined for their dissociated constituents by electrophoresis in SDS, the A fraction components must aggregate in the 4M urea solution that was used for fractionation by gel-filtration chromatography. In contrast, fractions B and C elute from the Sephadex columns at positions consistent with the molecular weights that were determined by SDS chromatography, thus indicating that 4M urea prevents their molecular association.

#### Ion-Exchange Chromatography of Fraction B

Separation of B-fraction proteins by ion-exchange chromatography on SEC was investigated since this procedure was previously successfully used to resolve gliadin proteins (12). Because derivatized B-fraction glutenin proteins are less soluble than gliadin proteins, it was essential to use a higher level of dissociating agent in the elution buffer than was necessary with gliadins. High concentrations of urea were avoided in order to reduce the possibility of cyanate formation and pH instability

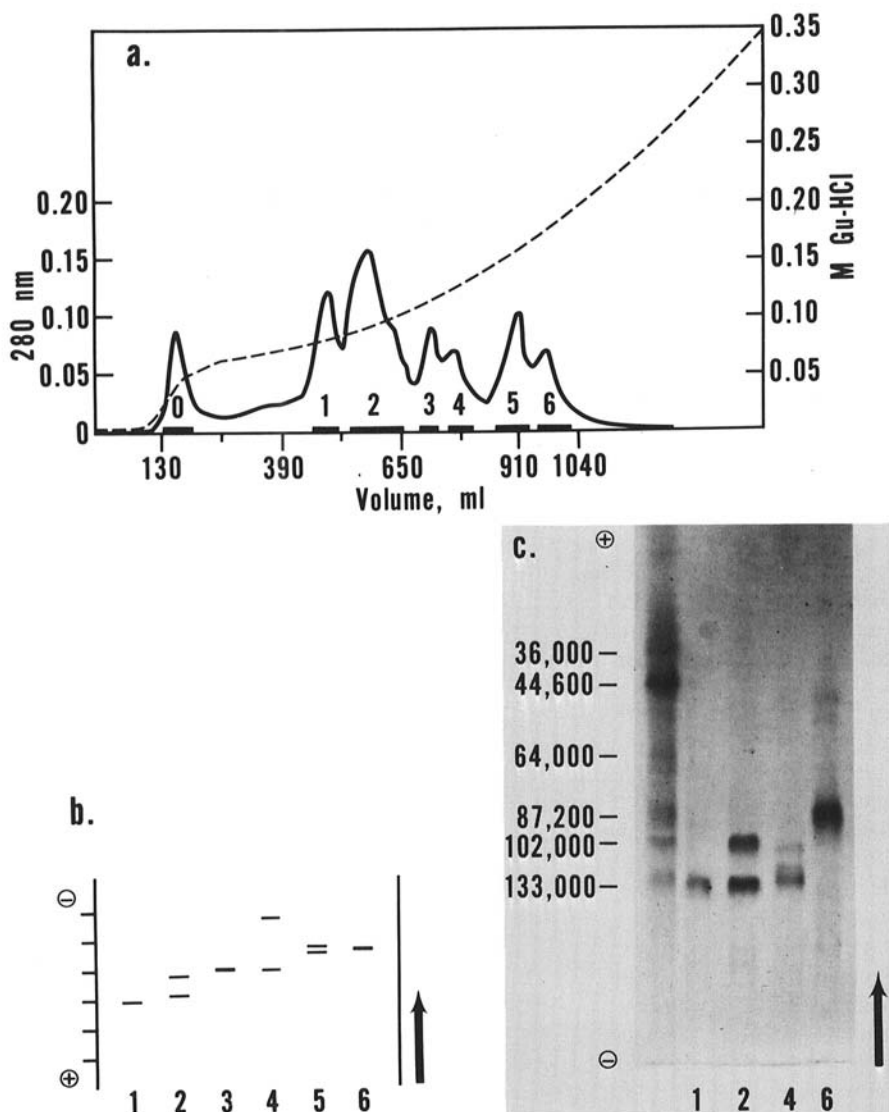


Fig. 4. a, Chromatographic separation of B fraction from Fig. 1 on sulfoethyl cellulose (SEC). Column:  $1.9 \times 50$  cm.; solvent: 3M dimethylformamide (DMF), 1M urea, 0.03N acetic acid, 0.005N HCl. Broken line shows salt gradient. Straight dark lines along the abscissa indicate fractions that were pooled. b, Starch-gel electrophoresis of fractions. c, SDS-gel electrophoresis of fractions 1, 2, 4, and 6. MW of reference proteins shown at left side.

due to urea decomposition. Therefore, the eluant contained 3M dimethylformamide and only 1M urea. In order to absorb the proteins strongly, the SEC column was operated at pH 2.8, attained by using 0.03N acetic acid and 0.005N HCl in the eluant. GHI was used to produce a salt gradient (from 0.05M to

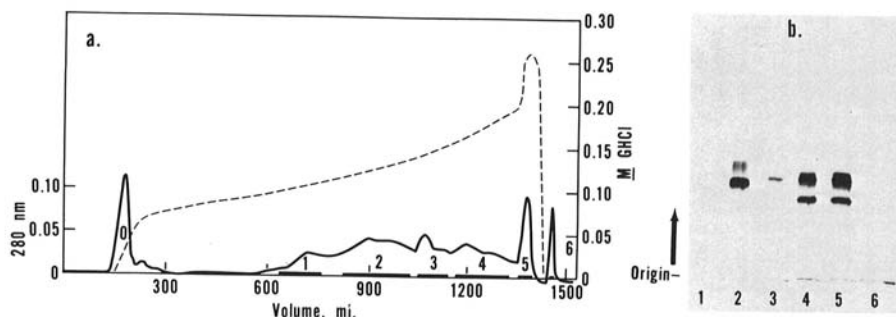


Fig. 5. a, Chromatographic separation of C fraction from Fig. 1 on SEC. Column:  $1.9 \times 50$  cm.; solvent: 3M DMF, 0.03N acetic acid, 0.005N HCl. Broken line shows salt gradient. Peak 6 eluted after the salt gradient with a pH 8.0 *tris*-buffer. Straight dark lines along the abscissa indicate fractions that were pooled. b, Starch-gel electrophoresis of fractions.

0.3M) since it is a good deaggregating agent and does not cause precipitation of the proteins on the column as NaCl might.

The chromatographic separation of fraction B on the SEC column and the electrophoretic analysis of separated fractions are given in Fig. 4. Seven distinct absorbance peaks appeared when the effluent contained the GHCl gradient shown in Fig. 4a. The components in the first peak (labeled 0) were not adsorbed on the SEC but were eluted at the void volume. The recovery of protein in the various fractions is given in Table I under "rechromatography." Starch-gel electrophoretic patterns of proteins in combined fractions composing peaks 1 through 6 are illustrated in Fig. 4b. The electrophoretic pattern of peak 0 is not shown in the figure, but its components migrated upon starch-gel electrophoresis to yield a few bands with mobilities lower than that of peak 1. These mobilities were similar to those obtained earlier for  $\omega$ -gliadins. Fractions 2, 4, and 5 each contain two electrophoretic components. However, in fraction 4 one of the components predominates. Fractions 1, 3, and 6 appear to be single components based on the starch-gel electrophoresis pattern (Fig. 4b). However, in the SDS-polyacrylamide electrophoresis pattern (Fig. 4c), peak 6 is resolved into two protein bands. The existence of only one component in peak 1 was confirmed by SDS electrophoresis. Peaks 2 and 4 also showed two different components upon SDS electrophoresis (Fig. 4c). It is likely that there are at least seven different components in the B fraction separated from Ponca reduced glutenin.

#### Fraction C

The C fraction was also chromatographed on the SEC column. It is the most soluble of the three fractions obtained by gel filtration, and the MWs appear to be similar to those of the gliadins. The eluant used for chromatography on SEC was the same as for the B fraction except for the elimination of urea. Samples applied were about 100 mg. and recovery is shown in Table I. Elution was attained by a salt gradient from 0.07M to 0.25M GHCl followed by pH 8.0, 0.1M *tris*-buffer (Fig. 5). Even with this nearly flat salt gradient, there was little indication from the chromatographic plot that the C fraction could be resolved into its constituent components (Fig. 5). However, by dividing the effluent exhibiting absorption at



280 nm. into five fractions and analyzing each by starch-gel electrophoresis, some degree of separation was noted. Evidently the charges on proteins of this group, as indicated by their electrophoretic behavior, are similar, thereby causing numerous proteins to be released from the column in close proximity.

Upon SDS-gel electrophoresis (Fig. 3), the C fraction appears to consist mainly of two MW fractions (about 38,000 and 43,000). Separating these two fractions would make further resolution more feasible on the SEC column.

Preliminary work with a Sephadex G-100 column showed that the C fraction can be partially resolved into three fractions when passed twice (recycled once) through a 90-cm. column (Fig. 6). Separation was greatest where the eluant was simply 0.05N acetic acid. In Fig. 6a, the first small peak, numbered 1, contained a mixture of proteins, presumably aggregated, since it came off at the void volume both without urea and with 2M urea in the eluant. The dashed portion of the next peak was recycled and was eluted again between 450 and 700 ml. It was divided into two fractions designated 2 and 3. Both fractions numbered 2 consisted of the higher-MW fraction and 3 was the lower-MW fraction on SDS gel (Fig. 6b).

The combined fractions 2 obtained as shown in Fig. 6 was chromatographed on the SEC column. The small quantity available did not allow any distinctive 280 nm. absorbance peaks, making division of the effluent as given in Fig. 7a rather arbitrary. Figure 7b shows the starch-gel pattern of these fractions. Fraction numbers 3 and 4 appear to be single components and 2, predominantly one with a trace of two others. The other fractions apparently consist of two components with a trace of another one. In Figure 7c, the SDS gel pattern of fraction 3 reveals a single MW band but that of 5 shows at least three different MW components.

The lower-MW fraction (No. 3 from Fig. 6) did not respond well to

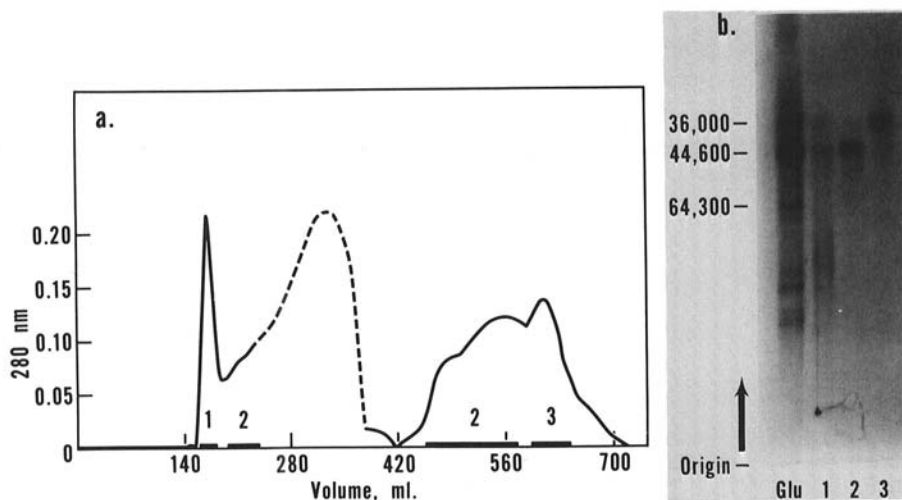


Fig. 6. a, Gel filtration of C fraction from Fig. 1. Column: Sephadex G-100, 2.5 × 85 cm.; solvent: 0.05N acetic acid; sample: 100 mg. Dashed area recycled. Solid line collected. Dark lines along the abscissa indicate fractions that were pooled. b, SDS-gel electrophoresis of fractions. MW of reference proteins shown at left side (from Ref. 6).

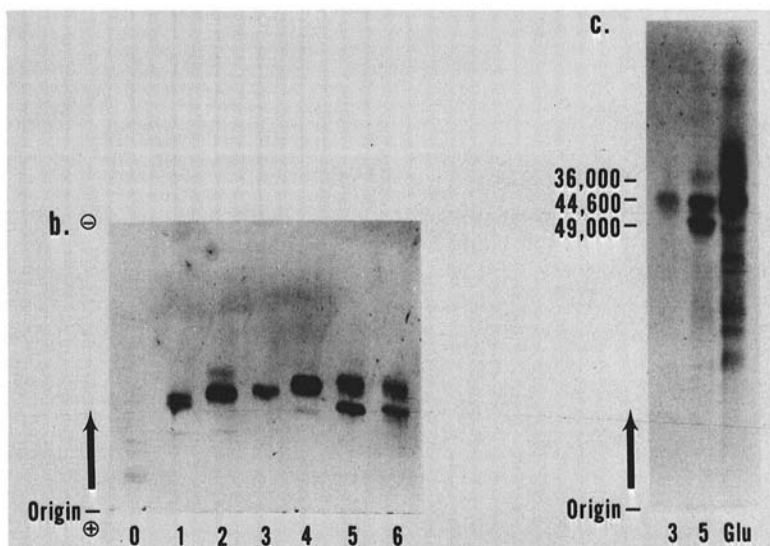
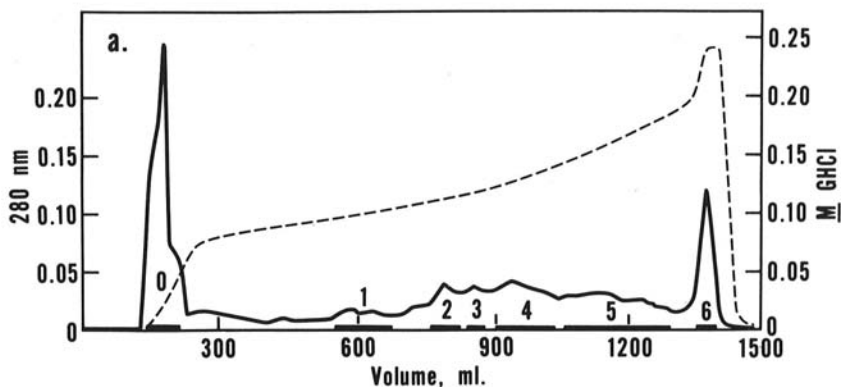


Fig. 7. a, Chromatographic separation of fraction 2 (HC) from Fig. 6a on SEC. Conditions same as Fig. 5. b, Starch-gel electrophoresis of fractions. c, SDS-gel electrophoresis of fractions 3 and 5. MW of reference protein shown at right side.

ion-exchange chromatography, and possibly consists of more than six components which are poorly resolved by this technique.

#### Fraction A: Separation

Fraction A is unusual because it does not give distinct bands on starch-gel electrophoresis in urea-containing buffer. On a SDS gel it migrates as streaky material with some low-MW subunits. Because aggregation was indicated, fraction A was chromatographed on a Sephadex G-200 column with 6M GHCl at pH 4.0 (Fig. 8). Recovery of the fractions is given in Table I. Part of fraction A still eluted at the void volume, but additional peaks occur at effluent volumes similar to those where fractions B and C elute with 4M urea solvent. Upon starch-gel electrophoresis, the

second peak gives a pattern similar to that of the C fraction in Fig. 1, but it stains less intensely on the gel. The third fraction streaks as it migrates in the starch gel ahead of the second fraction. On the SDS gel (Fig. 8b) the difference in migrations between these three fractions is considerable. Their mobilities provide additional evidence that fraction A, while it may be partially aggregated in a 4M urea buffer, contains components different from those of fractions B and C (Fig. 1).

#### DISCUSSION

Since previous work with reduced glutenins always indicated that they exhibited considerable aggregation and required concentrated urea or GHCl to promote dissociation, it may seem inconsistent to use 4M urea for gel-filtration separations. When 8M urea or 6M GHCl was used for the first fractionation on Sephadex G-200, part of A fraction was eluted with the B and C fractions making the latter fractions more complex and more difficult to further fractionate into individual components. Difficulties in flow rate and other column characteristics were encountered upon elution with these higher concentrations of urea or GHCl. Since the reduced glutenins were soluble in 4M urea, the lower concentration was tried. Both starch-gel and SDS-gel electrophoresis is showed that each of the three fractions contained different constituents. This method is also easily scaled up by using larger-diameter columns, can be run at high flow rates, and allows the column to be reused many times before repacking is necessary.

Evidently complexity of the glutenin mixture hampered earlier attempts to separate the proteins by ion-exchange fractionation. Both fractions B and C were eluted from the SEC column (Figs. 4 and 5) under nearly the same conditions. Thus, separation would be poor unless fractions A, B, and C were first separated by gel filtration.

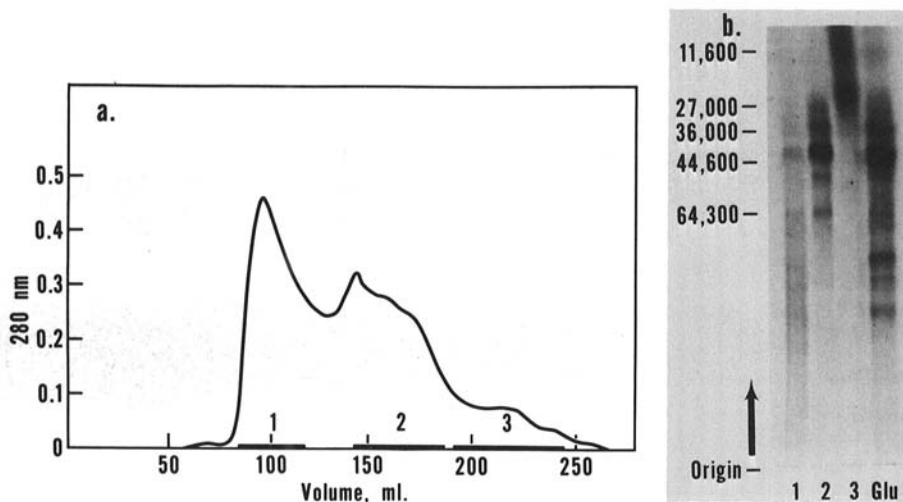


Fig. 8. a, Gel filtration of A fraction from Fig. 1. Column: Sephadex G-200, 2.5 × 72 cm.; solvent: 6M guanidine HCl, 0.01N acetic acid; 55-mg. sample. b, SDS-gel electrophoresis of fractions. MW of reference protein shown at right side.

These experiments demonstrate that the polypeptide chains which constitute glutenin are even more heterogeneous than previously shown by single techniques, such as starch gel (4), SDS electrophoresis (6), or gel filtration (5). Components that appear pure by a single criterion, such as electrophoretic mobility or MW, when subjected to a different fractionation tool, contained more than one constituent. Upon starch-gel electrophoresis, many components of reduced and alkylated glutenin resembled reduced gliadin in mobility (4). However, when subjected to gel-filtration separation, most of these glutenin subunits differ appreciably from gliadin in MW. As reported by others (5,6), our results verify that glutenin consists of at least three distinctly different groups of subunits; those in fraction C with low-MW proteins that may resemble gliadins, those in fraction B with higher MW, and those that are extensively aggregated in fraction A. The strong aggregation properties of the A-fraction subunits, the high-MW B fraction, and the manner in which these subunits are joined together by the disulfide bonds could be essential to the unusual characteristics of the native glutenin.

While the various procedures described here do not separate all the reduced glutenins into individual components, they do provide greatly simplified fractions; two appear to be pure by electrophoretic criteria. Using methods similar to those described here, additional individual components could be isolated. A detailed comparison of the amino acid composition of certain of these polypeptides to one another and to other wheat proteins is presented in the accompanying paper (13). Also, end-group analysis will be described which further indicates the purity of certain of the fractions. Such information may provide insight into the question of why glutenin subunits tend to form intermolecular disulfide bonds while the disulfide bonds of gliadin are intramolecular (4). The chromatographic fractionation system described here points out compositional differences in reduced glutenin not evident in other systems of separation. It is therefore being used at the Northern Laboratory to explore differences in glutenin structure among wheat varieties.

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